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Cellular and molecular aspects of p53 and an alternatively spliced protein, p53as, are being examined during cancer progression in both a mouse mammary and epidermal model. We report here that both p53 and p53as are expressed in cell lines and tissues of the mouse mammary model and, as previously reported, in epidermal cells. The half life of both proteins has been determined in asynchronous cells from both model systems. In mammary cells, but not in epidermal cells, p53as is longer lived than p53. Synchronizing untreated cells, either by serum deprivation or by density arrest followed by serum deprivation, has not proven reproducible enough for the proposed studies of cell cycle dependency of protein stability. Therefore, we will use selected drugs for a dual purpose: 1) to induce p53 levels and 2) to arrest cells at specific cell cycle stages. The p53as DNA binding sequence has been determined and found to be the same as the p53 sequence. However, we have noted sequences which may permit uncovering new p53 transcriptional target genes. We have begun studies of tissue specific p53- or p53as-associated proteins by the construction and isolation of fusion proteins of the glutathione-S-transferase protein and the C-terminus of either p53 or p53as and by purification of p53 and p53as proteins from a baculovirus system.

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Molly F Kules Martin 10-24-96

PI - Signature Date

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INTRODUCTION

The high frequency of p53 mutations in human cancers suggests that loss of wild type p53 function(s) plays a critical role in the etiology of carcinogenesis. Since the functions of p53 include regulation of cell proliferation, differentiation, and apoptosis, disruption of any one of these functions could result in cancer. Knowledge of the mechanisms underlying the normal functions of p53 will increase our understanding of cancer.

p53 protein contains multiple functional domains which include an N-terminal transcription activation domain, a central sequence-specific DNA binding domain, and a C-terminal non-specific DNA binding domain (Ko and Prives, 1996). The C-terminus also requires modification by phosphorylation, addition of a C-terminus-specific antibody PAb421, interaction with dnaK, truncation of the last 30 amino acids (Hupp et al., 1992; Hupp et al., 1993; Hupp et al, 1994), or binding to single-stranded DNA (Wu et al., 1995; Bakalkin et al., 1995; Bayle et al., 1995; Jayaraman and Prives, 1995; Hupp et al., 1995) in order for specific DNA binding to occur.

Our laboratory has observed an alternatively spliced form of p53 in normal mouse cells and tissues (Han and Kulesz-Martin, 1992; Kulesz-Martin et al., 1994). The alternative splicing results in a p53 protein, termed p53as, which contains 17 different amino acids at the C-terminus and is 9 amino acids shorter. *In vitro*-produced p53as is constitutively active for DNA binding as shown by gel shift assays (Bayle et al., 1995; Wu et al., 1994; Wolkowicz et al., 1995). In addition, p53as is preferentially associated with the G_2 phase of the cell cycle (Kulesz-Martin et al., 1994) while p53 is associated with G_1 and G_2 phases (Ko and Prives, 1996; Agarwal et al.,1995; Stewart et al., 1995). Recent evidence also indicates that p53as and p53 respond with different kinetics to DNA damage (Y. Wu, H. Huang, and M. Kulesz-Martin, submitted). These distinctions suggest different cellular functions for the two proteins.

The Specific Aims of this proposal were: 1) to examine the expression of p53 and p53as during mammary cancer development, 2) to determine the half life of p53 and p53as proteins and to detect associated proteins, and 3) to determine the oligomerization properties and DNA binding sequence specificity of p53as. The models to be used were a murine breast cancer model (Medina et al., 1993a; Medina et al., 1993b) and a mouse epidermal model developed in this laboratory. The mouse mammary epithelial cell lines are transplantable into syngenic mouse fat pads which produce mammary preneoplastic outgrowths (Medina et al., 1993a). The outgrowths produce tumors and include some which respond to estrogen. They have been designated TM for "tumor mammary". Established cell lines have been derived from the tumor outgrowths (Medina et al., 1993b). The murine clonal epithelial cell lineage is comprised of normal, initiated, benign, and malignant stages. As in the mammary model, epithelial cell types are available at various stages of preneoplasia and malignancy. Since all stages were derived from the same cloned parental cell, this model offers the same genetic lineage for all stages, thus ensuring controls for proliferative and differentiative states.

EXPERIMENTAL METHODS, RESULTS AND DISCUSSION

TASK 1 (Aim 1) Determination of p53 and p53as expression in the mouse mammary model. In the first year of this proposal, we determined the expression of p53 and p53as in 5 TM cell lines and observed that all of these cell lines contained mutated p53. We chose two of these lines, TM10 and TM3 (high passage), as representative of high- and low-tumorigenic states, respectively, for immunohistochemistry studies. Thin sections were made from frozen TM3 (high passage) and TM10 tissue in the RPCI Histology Laboratory and fixed in cold acetone for 15 minutes. Slides were washed in water and endogenous peroxidase quenched with 1.5% H_20_2 in methanol for 10 min at room temperature. After two 5 min washes with phosphate buffered saline (PBS) containing CAS™ Block (Zymed Laboratories, Inc.), the slides were

blocked with 5% goat serum in PBS for 20 min at room temperature and then incubated with 250 ng of the primary antibody overnight in a humidified container. Two 5 min PBS washes were followed by a 30 min incubation with the biotinylated secondary antibody at room temperature. The slides were washed twice in PBS for 5 min and incubated for 30 min in ABC reagent (Vectastain). After two 5 min washes in PBS, 3,3'diaminobenzidine terahydrochloride (DAB), an insoluble peroxidase substrate, was applied to the slides for 1-4 min, the slides were washed for 1 min in water, and counterstained with hematoxylin. The results of immunohistochemistry of frozen TM3 (high passage) and TM10 tissue are shown in Table 1. Two different tissue isolates of TM3 (high passage) were used for staining. All tissues show negligible nuclear reactivity using PAb421 (an antibody specific for the regular form of p53), PAb246 (an antibody which recognizes only the wild type conformation of p53/p53as), or PAb240 (an antibody which recognizes only a mutated conformation of p53/p53as). The polyclonal antibody CM-5 which recognizes both p53 and p53as was reactive with 60-70% of cell nuclei. The polyclonal antibody ApAs, which is specific for p53as protein, stained 10% of the cytoplasm in TM10 cells, 2% of the nuclei in one of the TM3 isolates and 50% of the nuclei in the other. All isotype controls for antibodies were negative. These results are different from the immunofluorescence staining of the TM cell lines in culture where PAb421, PAb240, and ApAs all stained approximately 30% of TM3 nuclei and PAb246 was negative, while untreated TM10 cells were essentially negative for all antibodies.

As described in the 1995 Progress Report, RT-PCR analysis showed that transcripts from both the regularly and alternatively spliced forms were present in all the examined TM cells. We have finished these studies by isolating RNA from TM3 (high passage) and TM10 cells and performing RT-PCR. Briefly, cells from a 150 mm plate were lysed in Ultraspec™ RNA solution (Biotecx Laboratories, Inc.), and 1 μg of total RNA used in a reverse transcription assay utilizing a random hexamer primer, and 30 units

of AMV-RT (Promega). Two µl of this reaction were used in 30 cycles of PCR containing 2 µM of each PCR primer (Han 1992) and 1 unit of *Taq* DNA polymerase (Boehringer Mannheim). Figure 1 shows that RT-PCR using TM3 (high passage) and TM10 RNAs produces a 517 bp product from p53 RNA and a 613 bp product from p53as RNA which has been seen in all the TM cells examined.

The determination of p53 and p53as protein and mRNA expression in TM 3 (high passage) and TM10 concludes Task 1.

TASK 2 (Aim 2, 3) Production and purification of p53as and p53 proteins in the baculovirus system. We previously reported that we had obtained murine p53 and p53as cDNAs in baculovirus vectors, made high titer virus stock, and had optimized the time after insect cell infection for maximum protein production at 2 days. At that time we were proceeding with antibody affinity purification with some difficulty both in obtaining relatively pure protein and in getting p53 or p53as to adsorb to the immunoaffinity column. We ultimately solved these problems but encountered very low yields of purified protein (typically ≤10 µg/50 ml culture), therefore we made histidine-tagged p53 and p53as proteins in baculovirus which could be purified by nickel affinity chromatography. A typical yield from this column using extract from a 50 ml culture is ≥215 µg of purified protein (data not shown). These his-tagged proteins may be used as an alternative method to identify p53- and p53as-associated proteins (see Task 6).

TASK 3 (Aim 2, 3) Determination of oligomerization properties of p53as. This was completed at the time of our last Progress Report and indicated using FPLC analysis that both p53 and p53as form dimers and tetramers. Gel shift experiments also showed that p53as oligomerizes with itself and with p53 (Wu et al., 1994).

TASK 4 (Aim 2) Determination of the half-life of p53as and p53 proteins. The increased stability of p53 and p53as proteins due to mutation in all the TM

cell lines would affect protein half-life studies. Therefore, we have elected to focus on 291 epithelial cells which are normal cells containing wild type p53 for these studies until a mammary cell line containing wild type p53 is available.

Determination of working conditions of centrifugal elutriation, cell synchronization, and protein half-life was in progress at the time of our 1995 report. These initial experiments indicated that centrifugal elutriation was useful for obtaining G_1 phase cells but did not allow the separation of S and G_2 in sufficient quantity for half life studies. However, we had achieved cell synchronization by serum starvation in 291 and 291.05RAT cells as assayed by tritiated thymidine uptake which would allow the isolation in sufficient quantity of cells throughout the cell cycle. More recent experiments with 291 and 291.05RAT which incorporated both flow cytometry data and tritiated thymidine uptake indicated that, although ^{3}H-thymidine uptake showed a growth arrest with serum starvation followed by resumption of growth with the addition of serum, flow cytometry indicated that most of the cells were not cycling and that a large percentage of the cells remained in G_1. These experiments were carried out by plating cells in 150 mm dishes and allowing them to grow to approximately 50% confluency, at which time the media was changed to 0.2% serum. After 48 hours, serum was replaced (5%) and cells allowed to grow for various times. Tritiated thymidine uptake was carried out by labeling a plate from a specific time point with medium containing 1 μCi ^{3}H-thymidine/ml for 1 hour at 37°C. The plate was then washed twice with PBS, 5 ml of 10% TCA was added and allowed to incubate at room temperature for 5 min, the plate washed once with PBS, and the cells lysed in 3 ml of 1 N NaOH for 30 min at room temperature with gentle rocking. A 100 μl aliquot of this was neutralized with 100 μl of 1 N HCl, 300 ml of H_2O was added, and the ^{3}H-thymidine incorporation quantified using a scintillation counter. At the same time cells were harvested for flow cytometry analysis using 3 ml of 0.25% trypsin for 5 min at 37°C. The cells were centrifuged, washed once with PBS, centrifuged, and resuspended in ~100

µl of PBS before fixing in 1 ml of ETOH. Approximately 1 X 10^6 fixed cells were centrifuged in a Beckman TG-6 at 1200 rpm for 5 min in a glass siliconized tube and resuspended in the dark in 500 µl of 1 mg/ml of bisbenzimide H 33342 fluorochrome (Hoechst dye) for ≥1 hour. Samples can be stored for 1-2 weeks at 4°C in the dark. Flow cytometry was carried out in the Flow Cytometry Laboratory at RPCI using a FACSTAR+ dual 5 watt argon laser system (Becton Dickinson Immunocytometry Systems). Data was analyzed using WinList software (Verity Software House, Inc.).

Figure 2 shows data obtained from 291 cells that were treated as described above. In Figure 2A, ^{3}H-thymidine uptake indicated growth arrest at the time of serum replacement (arrow) following 48 hours of serum starvation and an increase in growth occurring which peaks at 29 hours after serum replacement. The flow cytometry data from this experiment shown in Figure 2B also indicated growth arrest occurring after 48 hours of serum starvation with 80.55 % of the cells in G_1 but at 29 hours, 58.99 % of the cells were still in G_1 with 15.09 % of cells in S and 22.2 % in G_2 phases.

The protocol was changed so that 291 cells were grown to confluency, serum starved for 24 hours (0.2% serum), and then released from the growth block by replacing the serum (5%). In Figure 3A, ^{3}H-thymidine uptake indicated growth arrest after 24 hours of serum starvation (arrow) with resumption of growth beginning 16-24 hours after serum replacement. As before, the flow cytometry data (Figure 3B) also showed growth arrest after serum starvation with 84.42 % of the cells in G_1, and although some cells began to move into S phase at 16, 20, or 24 hours, a majority of the cells remained in G_1. For example, at 24 hours, 45.12 % of the cells were in G_1, 24.64 % in S, and 23.64 % in G_2.

The task of synchronizing the TM and epidermal cells in order to provide suitable amounts of cells for half-life studies were unsuccessful for the following reasons: 1) long cell cycle time prevented separating S and G_2/M by centrifugal elutriation, 2) high fraction of non-cycling cells after serum starvation-induced synchrony raised the background of G_1

cells at the times of maximum S or G_2/M in populations, and 3) low levels of p53 detectable by immunoprecipitation (the detection method required for these experiments). As a result, we were unable to obtain synchronous populations of either the mammary or the epidermal cells. We did, however, determine the half life of p53 and p53as in asynchronous TM3 and TM4 cells, as reported in 1995, and now report the initial results of half life studies in asynchronous 291 and 291.05RAT cells. These cells were grown to 70-80% confluency in 150 mm plates, the medium was changed to MEM without cysteine or methionine plus 5% dialyzed fetal calf serum and the plates were incubated for 30 min at 37°C. This medium was replaced with 6 ml of the same medium containing 1.2 mCi ProMix (^{35}S-cys, ^{35}S-met; Amersham). We have recently observed that labeling p53 and p53as at both cysteine and methionine residues increases their detection. After 1 hour at 37°C, the plates were washed in MEM, 5% fetal calf serum and 15 µg/ml methionine and cysteine. Plates were incubated at 37°C in the same medium for various chase times per time point. Cells were harvested by scraping in 10 ml PBS and the cell pellet was lysed in 100-200 µl of lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM PMSF, 5 mM leupeptin, 10 µg/ml pepstatin A) for ≥1 hour at 4°C. The lysate was cleared by microcentrifugation at 13,000 rpm for 15 min and the supernatant precleared by adding 20 µl of a protein A slurry (1:1 in lysis buffer). This was rocked for 1.5 hours at 4°C. Immunoprecipitations were carried out by rocking overnight at 4°C with equal TCA-precipitable counts in a total volume of 450 µl containing lysis buffer and 2 µg of ApAs or 20 µg of PAb421 antibody. Twenty-five µl of protein A slurry was then added and the mixture rocked at 4°C for 2 hours. Immune complexes were collected by centrifugation, washed 3 times in 1 ml of lysis buffer containing proteinase inhibitors, and resuspended in 25 µl of 1X Laemmli loading buffer before heating at 95°C for 5 min and loading on a

7.5% polyacrylamide gel. Figure 4A shows immunoprecipitations from 291.05RAT cells and Figure 4B is a graph generated from densitometric analysis of the autoradiogram. The half life of p53 or p53as proteins in 291.05RAT cells was approximately 2.7 hours and 0.7 hour, respectively. These cells had been treated with the DNA damaging agent genistein (60 μM) 3 hours prior to labeling cells to increase amounts of p53 and p53as. The half life of p53 or p53as proteins in similarly treated asynchronous 291 cells was approximately 3 hours and 2.5 hours, respectively (data not shown). Genistein has been shown to increase the stability of p53 (Y. Wu, H. Huang, and M. Kulesz-Martin, submitted) and most likely accounts for the longer half life of both proteins (≤3 hours) since both 291 and 291.05RAT contain wild type proteins which usually have a shorter half life (Ko and Prives, 1996). In any event the half lives in 291.05RAT are shorter than those previously reported in untreated TM3 and TM4 cells which were determined to be ≥5 hours. In those cells the long half life is indicative of a mutant p53 molecule which was corroborated by immunofluorescence. Another difference between the mammary and the epithelial cell lines is that mutant p53as had a slightly longer half life than p53 in TM3 and TM4 while in 291 and 291.05RAT, the wild type p53as had a slightly shorter half life than p53. This may reflect differences in cell types or between wild type and mutant proteins.

We have been unable to synchronize cells using serum starvation to examine the half life of p53 or p53as during the stages of the cell cycle. In the following year we will explore the use of cell cycle arrest drugs to achieve synchronization. The G_1 arrest agent, aphidicolin, will be used to obtain cells at this phase of the cell cycle while higher doses of genistein, a G_2 arrest agent, will be used to obtain G_2-phase cells. Our aim is to block cells in G_1 or G_2 for determination of half life of p53 and p53as.

Task 5 (Aim 3) Determination of a p53as-specific DNA binding site(s) and assay of p53as and p53 binding activities. p53 functions as a transcription factor and is able to affect the expression of genes involved in cell proliferation, differentiation, and apoptosis. DNA binding sequences which respond to p53 have been identified both in

upstream promoter regions and in the introns of specific genes. Studies have delineated a consensus p53 binding sequence consisting of two repeats of 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' (Kern et al., 1991, El-Deiry et al., 1992 Funk et al., 1992) which more recently has been refined to two repeats of 5'-PuGPuCATGPyCPy-3' (Thukral et al., 1995). Given the distinct functional properties of p53 and p53as, one explanation could be differences in their DNA binding due to different C-termini. Therefore, the p53as DNA binding sequence was determined to test this possibility.

p53as-specific DNA binding sequences were identified using cyclic amplification and selection of targets (CASTing) (Wright et al., 1991; Brown and Baer, 1994). A 96-mer single-stranded DNA was synthesized by the Biopolymer Laboratory (RPCI) which contained an 18 nucleotide primer site (5'-ATACCAGCTTATTCAATT-3') followed by 60 random bases and ending with another 18 nucleotide primer site (5'-AGATAGTAAGTGCAATCT-3') (Crameri and Stemmer, 1993). This was made double-stranded by annealing a primer complementary to the 3' 18 nucleotides in a 1:1 molar ratio followed by primer extension using 5 units of *Taq* DNA polymerase in a 100 µl reaction mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, and 200 µM of each deoxynucleoside triphosphate. This was placed at 94°C for 15 min, 60°C for 1 min, and 72°C for 30 min

To bind p53as-specific antibody to magnetic beads, 20 mg of sheep anti-mouse IgG coated magnetic beads (Dynabeads 280, Dynal Inc.) were washed in 5 ml of PBS/0.1% BSA three times and resuspended at 30 mg/ml in the same solution. BC4-17, a monoclonal antibody specific for p53as (0.5 mg), was added to a 100 µl sample of the washed beads and rocked for 2.5 hours at 4°C. The reaction was washed 4 times by adding 1 ml of PBS/0.1% BSA, incubating at 4°C for 30 min, and recovering the beads with a magnet. The final resuspension was in PBS/0.1% BSA at 30 mg/ml.

The association of the polymerized double-stranded DNA to the antibody-coated magnetic beads was carried out by mixing 5 μg of the DNA with 4 μl of *in vitro* translated p53as protein (Wu, 1994) and 2 μl of buffer (20 mM Hepes pH 7.6, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 10 mg/ml BSA). This was incubated at room temperature for 20 min after which 10 μl (300 μg) of the antibody-coated magnetic beads were added and the mixture gently agitated for 60 min at room temperature. The volume was increased by the addition of 100 μl of PBS/0.5% NP-40/0.1% BSA and the magnetic bead/p53as/DNA complexes retrieved with a magnet. After three washes in the PBS/NP-40/BSA solution, the complexes were washed once with PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.9 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate) and resuspended in 40 μl of PCR buffer.

To amplify the bound DNA sequences, 30 μl of this resuspension was added to 1.2 ug of primers complementary to the 18 nucleotide primer sites and subjected to one cycle of 80°C for 3 min, 94°C for 2 min, and 46°C for 5 min after which 2.5 units of *Taq* DNA polymerase and 0.5 units of Perfect Match (Stratagene) were added. Thirty amplification cycles were carried out (94°C for 1 min, 46°C for 1 min, and 72°C for 1 min) followed by a final 10 min at 72°C. Fourteen μl of the PCR reaction was used to initiate the next CASTing cycle. Six cycles were carried out with the last 4 cycles containing 10 μCi of α^{32}P-dCTP.

Aliquots of PCR products from the 6 CASTing cycles were separated on an agarose gel as shown in Figure 5. The expected 96 basepair DNA can be seen in all lanes and is indicated with an arrow. Increasing amount of higher molecular weight molecules, commonly seen in successive cycles with this technique attributable to futile cycling due to

limiting reagents, are seen to be the highest in cycle 6. Primer dimers are the lowest molelcular weight bands observed.

DNA binding assays were carried out on ice for 30 min in 15 µl volumes that contained 3 µl of an *in vitro* protein reaction, 10 µg poly [d(I-C)], 0.1 mg/ml BSA, and 0.1 µl of a labeled PCR reaction (~100,000 cpm) or 20,000 cpm of an end-labeled DNA fragment in a buffer composed of 20 mM Tris-HCl pH 7.2, 80 mM NaCl, 1 mM EDTA, 5 mM DTT, 4% glycerol, and 0.1% Triton X-100. Assays containing p53 also contained 100 ng PAb421. A p53 DNA binding consensus sequence (El-Deiry et al., 1992) was used for a positive control. Reactions were run on 4% nondenaturing polyacrylamide gels in 0.5X TBE at 4°C, the gel dried and products visualized by autoradiography.

Gel shift assays using aliquots of labeled PCR reactions from cycles 3, 4, and 5 (the same reactions seen in Figure 5) and *in vitro* translated p53as protein are shown in Figure 6. The arrow indicates the shifted DNA containing p53as (lanes 1-3) which migrates similarly to p53as protein bound to a consensus p53 binding sequence (lane 4). The faster migrating band present in this lane and in lane 6 was non-specific as it was not supershifted in the presence of ApAs, the p53as-specific antibody (lane 9).

An aliquot of cycle 5 was used to produce molecular clones of the putative p53as binding sequences to decrease the possibility of introducing nonspecific higher molecular weight DNA into the ligation reaction that were present in cycle 6 . Plasmid DNA from 64 transformants was isolated and used in PCR reactions containing α^{32}P-dCTP. The labeled PCR reactions were then used in gel shift assays with *in vitro* translated p53as. Fourteen of these clones were positive for p53as binding and were sequenced and the sequences obtained shown in Table 2. The flanking primer sequences were present in all clones and are not shown. Three clones, 64/38, 28/24, and 59/42, were found twice. PCR artifacts were found in several clones but are unlikely to affect the analysis: clones 28/24 and 36

had two randomized 60 base pair sections separated by multiple PCR primers, several clones contained more than one primer at one or both ends.

The fourteen clones positive for p53as binding were also tested for binding to p53. All did bind to *in vitro* translated p53 but, as expected, only in the presence of PAb421 (data not shown). An autoradiogram of gel binding assays with representative CASTing sequences and p53 or p53as are shown in Figure 7 and indicates binding by both proteins. The specificity of p53as binding was tested by adding ApAs to a binding reaction. All of the 14 sequences were examined and showed a supershift under these conditions as shown in Figure 8

All of the 11 non-duplicated p53as binding sequences contained the tetranucleotide CATG and 8 of the 11 contained two or more CATG motifs (shown in bold print in Table 2). The p53 consensus sequence (5'PuPuPuCATGPyPyPy-3') (El-Deiry et al., 1992, Funk et al., 1992; Thukral et al., 1995) also contains 2 CATG motifs. Only two sequences, 13 and 64/38, fit the consensus in regard to three purines followed by CATG followed by three pyrimidines. Neither 13 nor 64/38 fit the refined consensus requirement for "Gpu" immediately upstream and "PyC" immediately downstream of the CATG (Thukral et al., 1995). Two sequences, 6 and 14, did adhere to this requirement if only the three nucleotides upstream of the first CATG and the three nucleotide downstream of the second CATG are considered (indicated +/- in column R/Y, Table 2). Three sequences, 18, 21, and 62, contained only 1 CATG although 18 and 62 had CTTG or CTAG, respectively, which is consistent with the less stringent consensus sequence (5'-PuPuPuC(A/T)(T/A)GPyPyPy-3') (El-Deiry et al., 1992; Funk et al., 1992). Sequence 21 had only 1 CATG and did not have an additional C(A/T)(T/A)G. Since eight of the 11 sequences deviated from the consensus sequence, we designed synthetic oligonucleotides to test requirements for p53as binding.

Several oligonucleotides and their complement sequences were synthesized, annealed, and used in DNA binding assays with *in vitro* translated p53as or p53. These

synthesized sequences are shown in Table 3. Each contains a central test sequence flanked by a common unrelated sequence (Thukral et al., 1995). p53as binding correlated with p53 binding (in the presence of PAb421), so both are shown together. One copy of 5'-PuGPuCATGPyCPy-3' was insufficient to confer p53as binding, but two copies of CATG with 5 or 6 intervening nucleotides was sufficient for binding. Internal symmetry or consensus flanking sequences did not appear to play a role. This was confirmed using WAF-1 in which the native WAF-1 CATG and intervening sequences are present without the native flanking sequences, and mWAF-1, in which internal and flanking sequences are altered. Binding was not observed when either 0, 1, or 3 intervening nucleotides were present indicating that the CASTing sequences separated by 0 to 3 nucleotides found in 6, 14, and 36 were not sufficient for the observed p53as binding and that regions within the longer stretch between CATGs (ranging from 23 to 70 nucleotides) must be involved. DNA sequences containing two copies of 5'-PuGPuCATGPyCPy-3' in which the CATG sequences are separated by 6 or 29 nucleotides (2CATG6 and 2CATG29) were bound by both proteins, supporting the results with CASTing sequences 6, 14, 28/24, and 36.

These results did not answer whether two CATG motifs separated by more than 29 nucleotides are sufficient for p53as binding. In addition, the DNA designated CCCGGG for the sequence adjacent to CATG in sequence 62 also found in the p53 binding sites of p53con (Funk et al., 1992) and the cyclin G promoter (Okamoto and Beach, 1994), was not bound by p53as or p53. The requirement for additional unidentified sequence(s) for binding must also be invoked for sequences 21, which has only 1 CATG, and 59/42, which has only the 2 CATG sequences separated by 3 nucleotides. Therefore, we examined each of the CASTing sequences for homology with other reported p53 binding sequences that are shown in Table 4.

All of the sequences except sequence 6 contained one or more copies of motifs found in reported p53 binding sequences. Homologous regions from these reported sequences and the sequences retrieved by CASTing with p53as protein are underlined in

Table 4 and summarized in Table 5. Of the sequences tested, all bound p53as as indicated in Table 4. It was striking that 5 out of 11 of the p53as binding sequences contained a region of homology to the novel sequence MgBH6 (Foord et al., 1993) (see the column headed * in Tables 2 and 4). As described in that report, this p53 binding sequence was obtained from mouse fibroblast DNA and was not found at the time in known genes. Furthermore, the entire region shown was protected by p53 protein in DNA footprinting assays and conferred activation of a reporter gene in transcription assays in cells (Foord et al., 1993). The segments of the MgBH6 sequence retrieved include TTGGC, present in 3 CASTing sequences as an inverted repeat. Thus the CASTing results may further define the p53 binding residues of this repeated sequence.

We performed FASTA searches of the GenBank and EMBL databases using several combinations of the motifs found in the p53as retrieved sequences or shared with known p53 binding sequences. The identical consensus-like sequence 5'-GAACATGTCCGGACATGTTC-3' independently retrieved in sequences 13 and 64/38 was found as a homologous match to several genes including AP-1 (L16546) and human ICAM (J03132). An inverted repeat of TTGGC of the published sequence MgBH6 (Foord et al., 1993), CGGTT-TTGGC, also present in 21, 28/24, and 59/42, was found in thrombospondin (J05605), the human CMV enhancer region (M64944), and a human homologue of dnaJ (D13388).

We next tested the affinity of p53as and p53 for DNA binding. Two of the synthesized DNAs that were positive for binding, 2CATG6 and 2CATG29, were examined for differences in affinity between *in vitro* translated p53 and p53as. Gel shift assays that utilized 2 ng of ^{32}P-labeled consensus sequence probe were subjected to increasing amounts of 2CATG6 or 2CATG29 unlabeled probe. Figure 9 shows the autoradiogram from one of two such experiments. The affinity of both proteins for 2CATG6 was greater than for 2CATG29 since 2 ng of cold 2CATG6 probe was able to compete all binding of

the labeled consensus sequence probe while 10 ng of cold 2CATG29 did not completely compete binding to the labeled probe for either protein.

TASK 6 (Aim 2) Comparison between p53as- and p53-associated proteins. We began the comparison of p53as- and p53-associated proteins by constructing the C-termini of both proteins in the expression vector pGEX-2TK (Pharmacia) to generate glutathione-S-transferase (GST) fusion proteins. These proteins contain approximately 225 amino acids from the GST protein (including a thrombin cleavage site) fused to either the last 91 amino acids of p53 or the final 84 amino acids of p53as.

The p53as protein has been produced in bacteria by subculturing a culture grown overnight in LB/100 μg/ml ampicillin 1:100 in 2 liters of LB/ampicillin. Cultures grown until OD_{600}=0.025-0.060 were exposed to 200 μM isopropyl β-D-thiogalactopyranoside (IPTG), and allowed to grow for 5 hours. The cells were harvested at 5,000 rpm for 5 min in a GSA rotor and the pellet(s) frozen at -80°C.

Stored pellets will be lysed using lysozyme and 2% sarkosyl to solubilize the proteins and then allowed to associate with a 50% slurry of glutathione beads (Pharmacia) at 5 to 7 mg protein/ ml of slurry for 3 hours at 4°C with gentle agitation. Beads containing either p53, p53as or the GST protein alone will be used in protein binding assays with protein extracts from 291 or TM10 cell lines. Briefly, bead-protein complexes will be incubated with cell extracts from 291 or TM10 cells and washed with increasing salt concentrations. Each batch elution will be analyzed by SDS-PAGE followed by Coomassie blue staining. Proteins which bind to GST will be eliminated from further consideration. Unique proteins bound by either p53as or p53 from 291 or TM10 extracts will be microsequenced. Similar procedures have been carried out in the laboratory and provided suitable amounts of proteins for microsequencing.

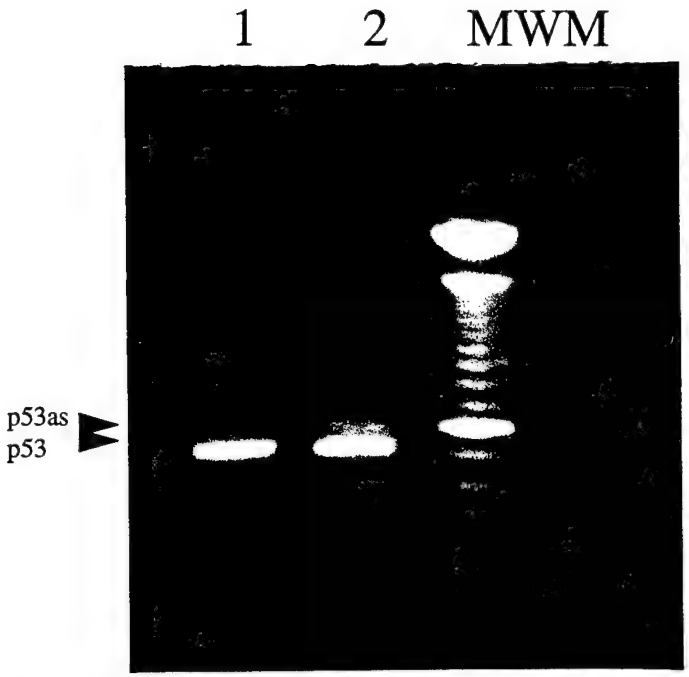
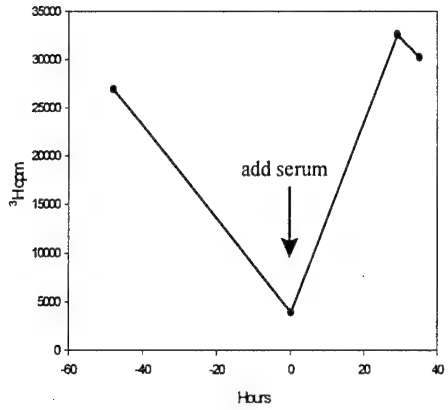
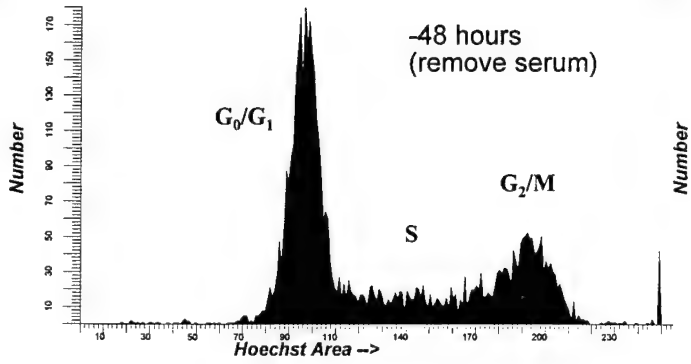


Figure 1. RT-PCR analysis of TM3 (high passage) and TM10 RNA. Arrows indicate the positions of the 517 bp product from regularly spliced p53 (p53) and the 613 bp product from alternativelyspliced p53 (p53as). Lane 1, TM3 (high passage); lane 2, TM10, MWM=100 bp ladder molecular weight marker.

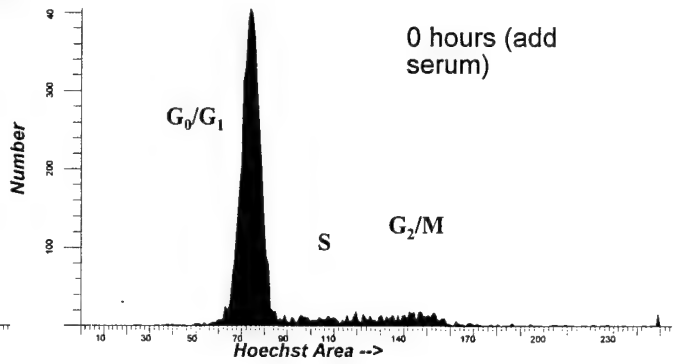
A)



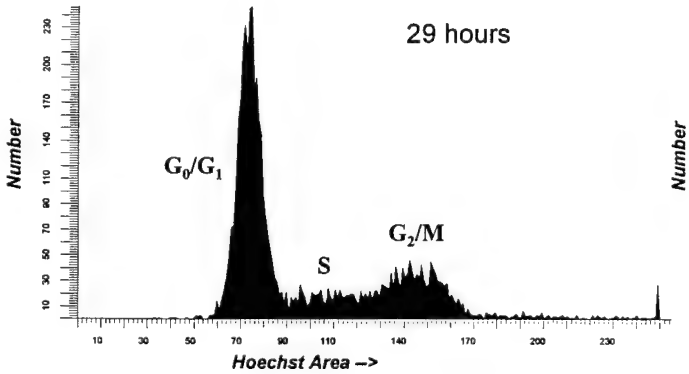
B)



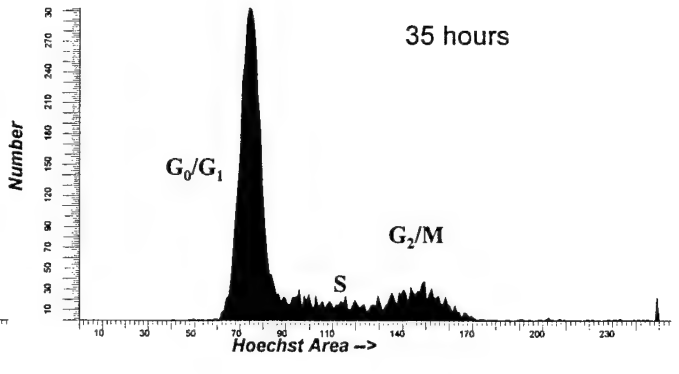
Percentage of Cells	
G_0/G_1	52.08
S	20.56
G_2/M	24.69



Percentage of Cells	
G_0/G_1	80.55
S	10.91
G_2/M	4.97



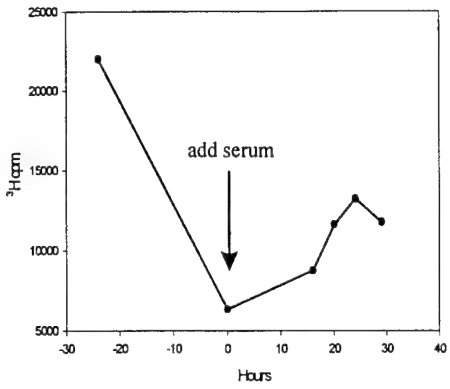
Percentage of Cells	
G_0/G_1	58.99
S	15.09
G_2/M	22.20



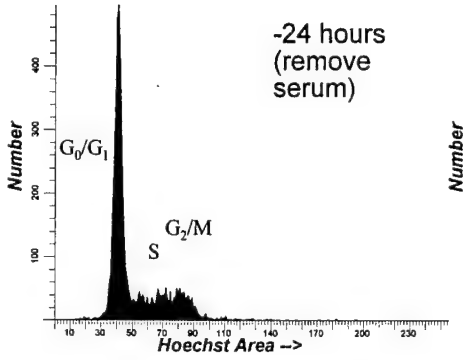
Percentage of Cells	
G_0/G_1	65.91
S	16.76
G_2/M	15.05

Figure 2. Synchronization of 291 cells grown to 50% confluency and serum starved (0.2%) for 48 hours. A) ^{3}H-thymidine uptake; serum was added at 0 hours as indicated. B) Flow cytometry data of the same time points shown in A; X-axis is Hoechst-labeled DNA, Y-axis is the number of counted cells. Cell cycle phases are indicated. The percentage of cells in G_0/G_1, S or G_2/M are indicated below each graph.

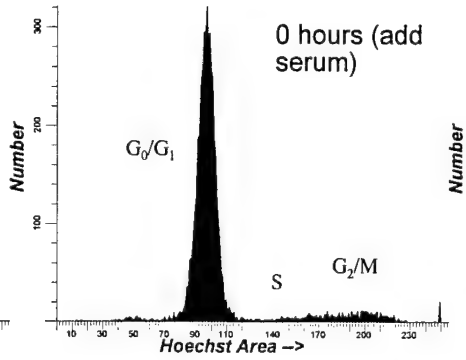
A)



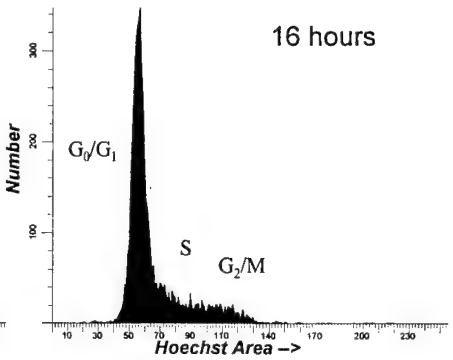
B)



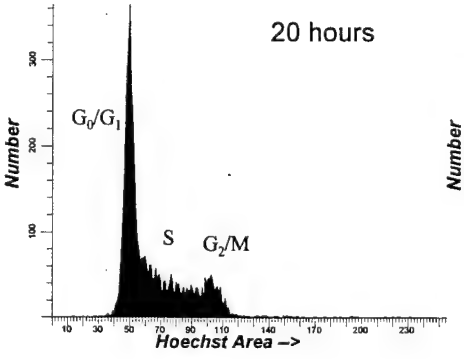
Percentage of Cells	
G_0/G_1	63.96
S	16.30
G_2/M	16.06



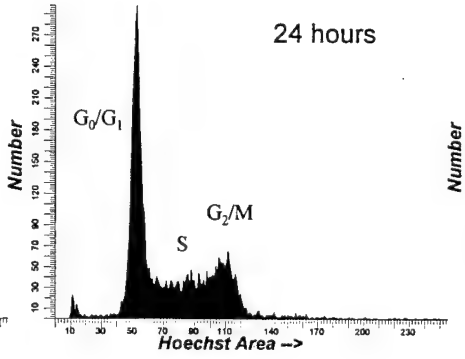
Percentage of Cells	
G_0/G_1	84.42
S	6.14
G_2/M	6.30



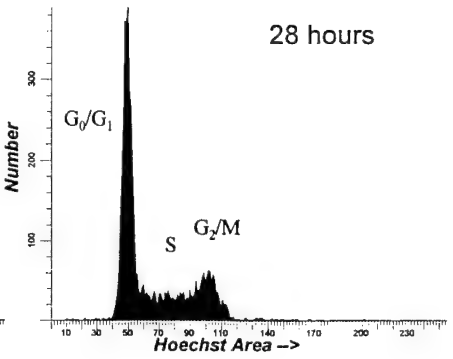
Percentage of Cells	
G_0/G_1	71.14
S	17.74
G_2/M	9.46



Percentage of Cells	
G_0/G_1	51.32
S	32.50
G_2/M	13.60



Percentage of Cells	
G_0/G_1	45.12
S	24.64
G_2/M	23.64



Percentage of Cells	
G_0/G_1	54.94
S	21.78
G_2/M	20.56

Figure 3. Synchronization of 291 cells grown to 100% confluency and serum starved (0.2%) for 48 hours. A) ^{3}H-thymidine uptake; serum was added at 0 hours as indicated. B) Flow cytometry data of the same time points shown in A; X-axis is Hoechst-labeled DNA, Y-axis is the number of counted cells. Cell cycle phases are indicated. The percentage of cells in G_0/G_1, S or G_2/M are indicated below each graph.

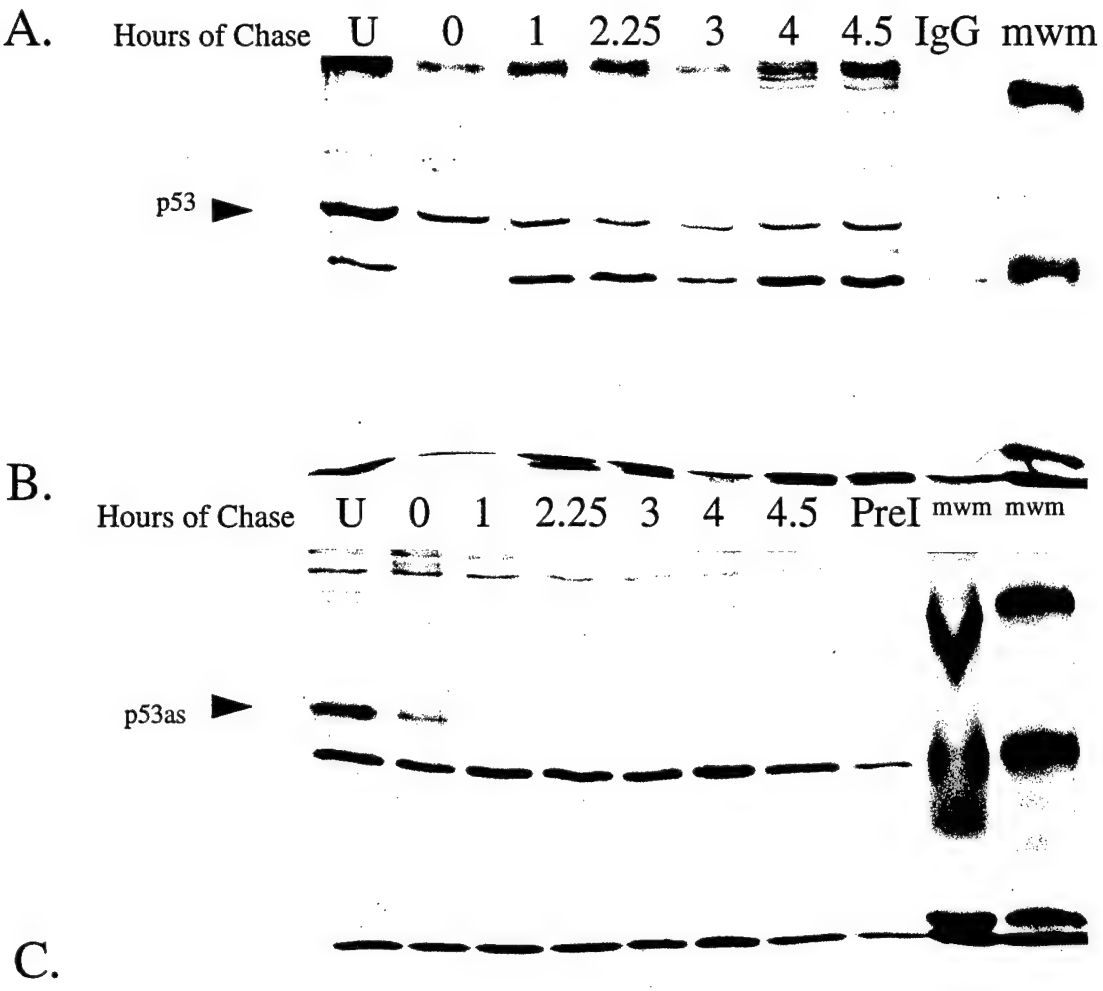


Figure 4. Half life analyses of genistein-treated, asynchronous 291.05RAT cells. A) Autoradiogram PAb421 immunoprecipitated p53, U=untreated, IgG=IgG used for immunoprecipitation, mwm=protein molecular weight markers; B) Autoradiogram of ApAs immunoprecipitated p53as, U=untreated. PreI= preimmune serum, mwm=protein molecular weight markers; C) Densitometric analysis of autoradiograms. Closed circles=p53; open circles=p53as. Half lives of p53 (2.7 h) and p53as (0.7 h) are shown.

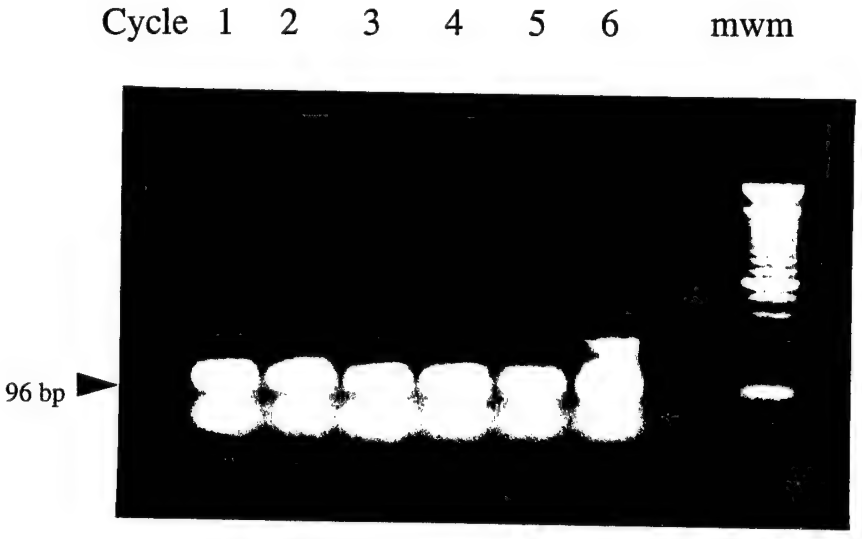


Figure 5. Agarose gel electrophoresis of the PCR products from CASTing cycles 1-6. Aliquots of PCR-products were separated on a 1% agarose gel. CASTing cycles 1-6 and the 100 bp ladder molecular weight marker (mwm) are indicated. The arrow indicates the expected 96 bp amplified DNA.

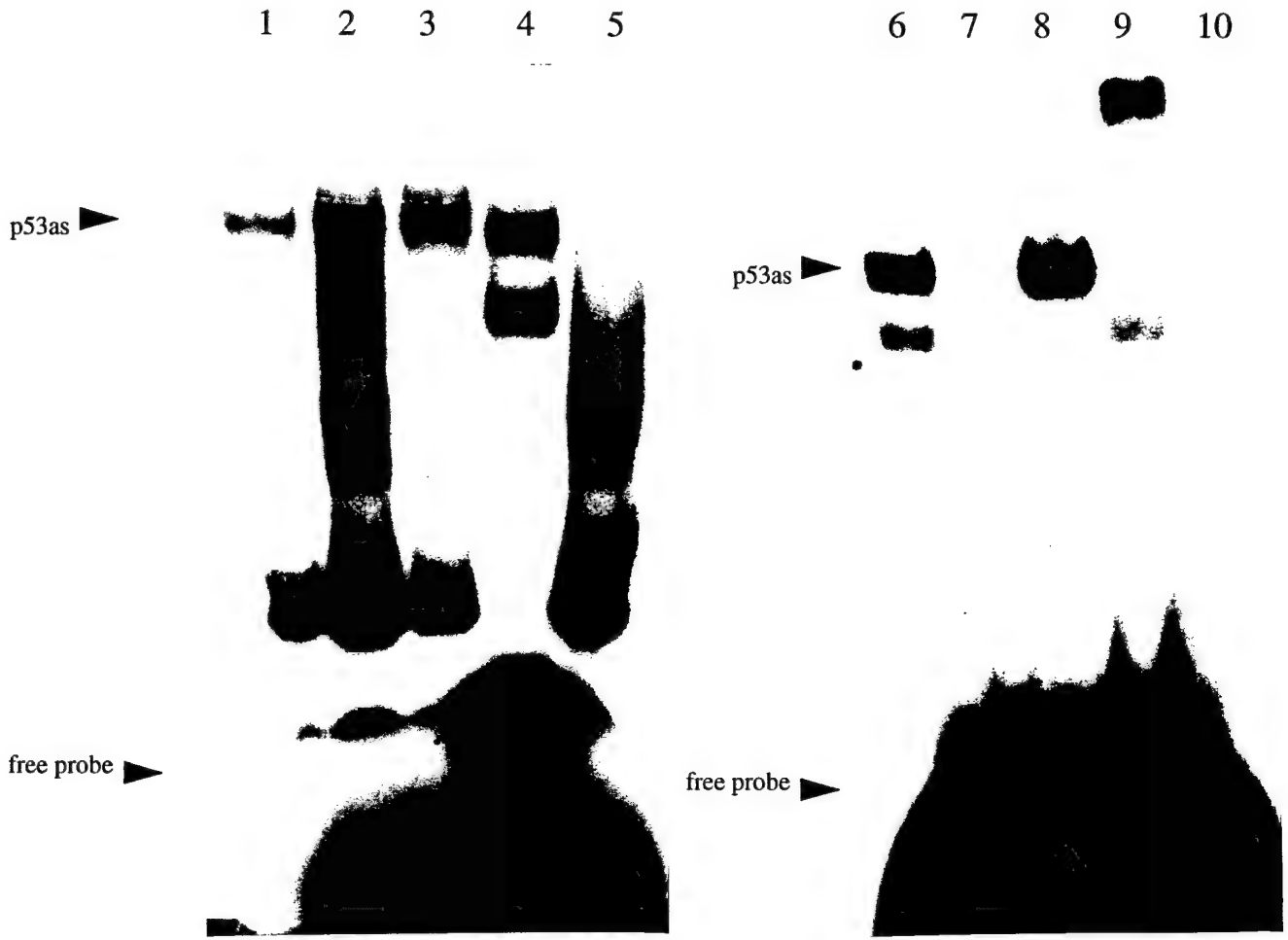


Figure 6. Autoradiogram of gel shift assays using CASTing cycles 3, 4, and 5. Aliquots of α^{32}P-PCR products from cycles 3, 4, and 5 were used in gel shift assays with *in vitro* translated p53as. Lane 1, cycle 3; lane 2, cycle 4; lane 3, cycle 5. Lanes 4-10 contain γ^{32}P-p53 consensus probe and *in vitro* translated p53as. Lanes 4 and 6,no addition; lanes 5 and 10, reticulocyte lysate used inthe *in vitro*translation; lane 7, plus cold competitor p53 consensus DNA; lane 8, plus cold competitor mutated p53 consensus DNA; lane 9, plus ApAs antibody.

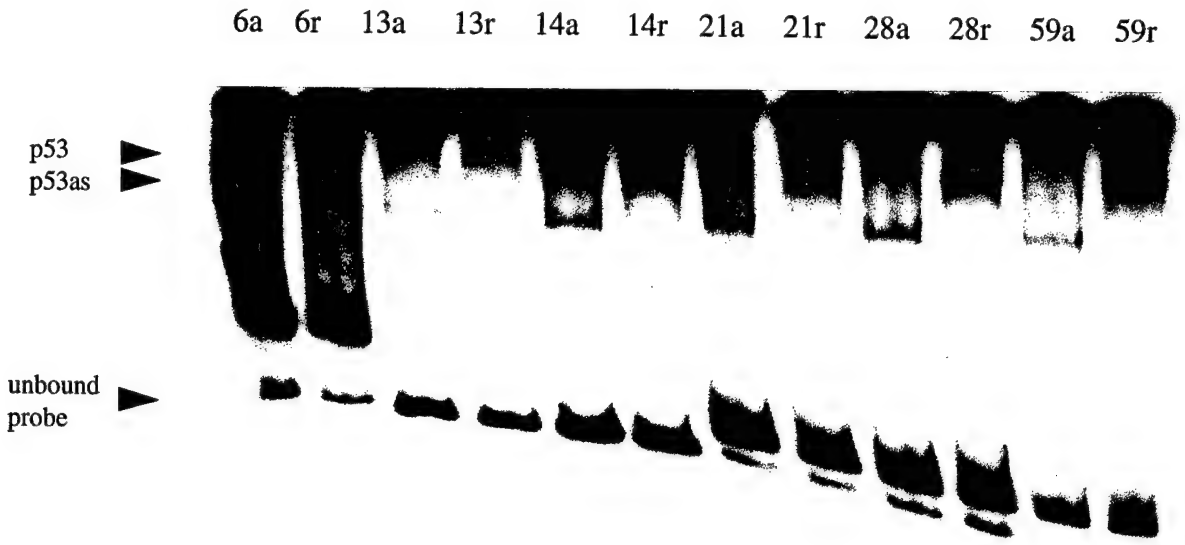


Figure 7. Autoradiogram of gel shift assays containing *in vitro* translated p53 or p53as proteins and aliquots of α^{32}P-PCR products from representative CASTing sequences. The number of each CASTing sequence and the protein present in the binding assay are indicated, a= p53as protein, r=p53 protein. The arrows indicate p53- or p53as-shifted sequences. Unbound probe is indicated. Control binding to end-labeled p53 consensus sequence was done as shown in figure 6.

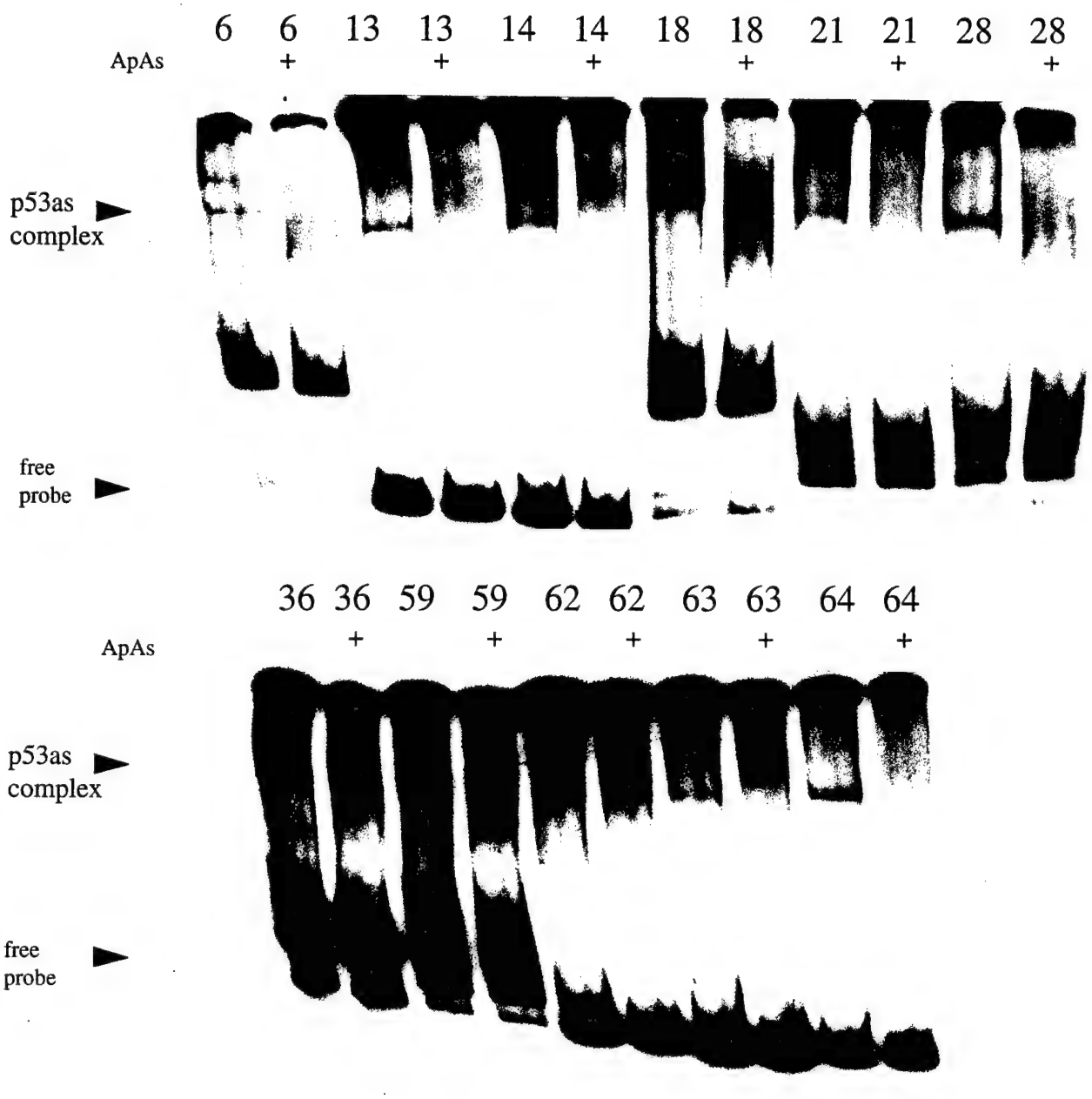


Figure 8. Autoradiogram of ApAs supershifting p53as binding of CASTing sequences. Assays were done in the presence (+) or absence (-) of ApAs antibody. p53as complexes are indicated with an arrow. Free probe is shown.

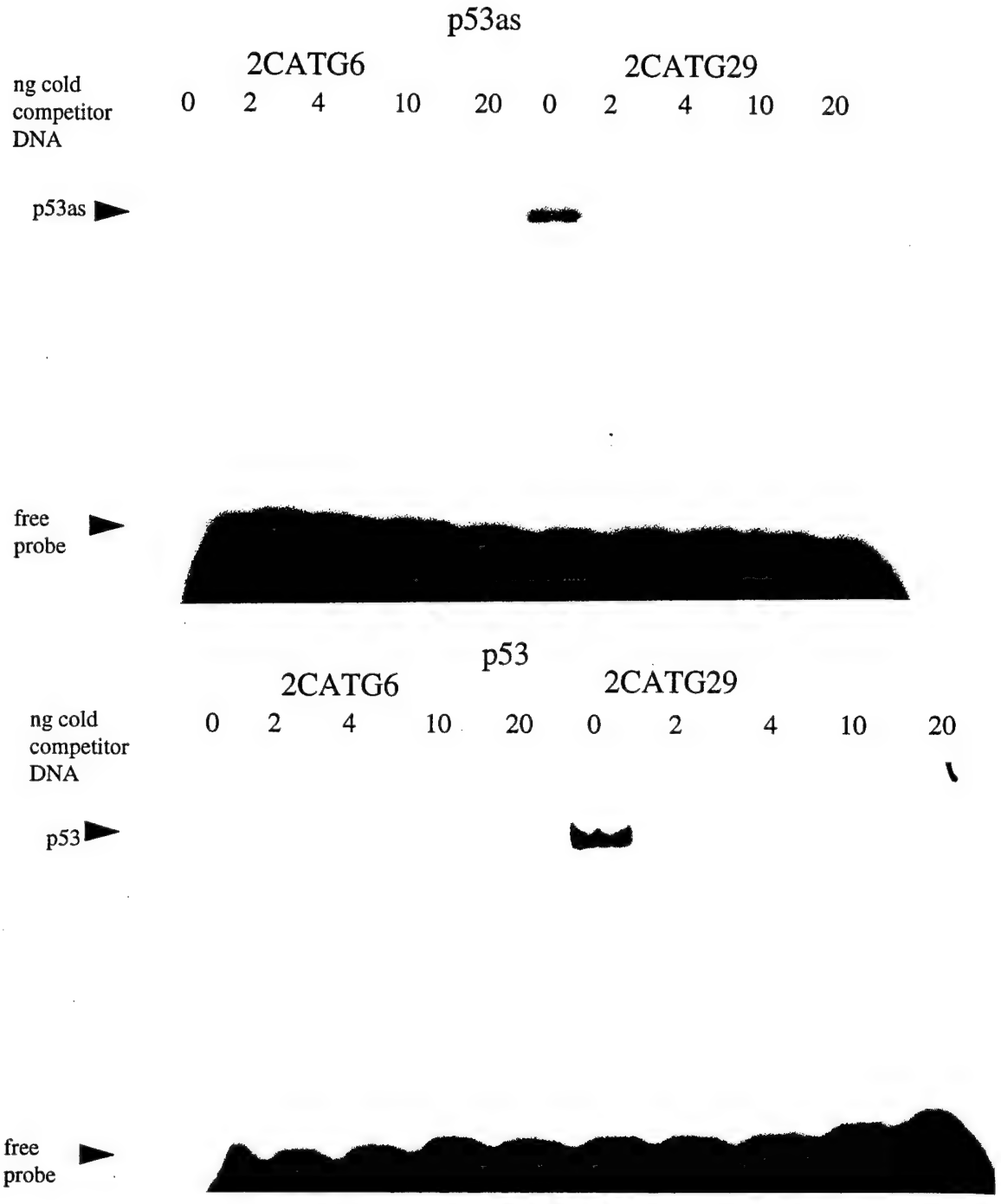


Figure 9. Autoradiogram of gel shift assays containing *in vitro* translated p53 or p53as, γ^{32}P-p53 consensus probe, and increasing amounts of cold competitor 2CATG6 or 2CATG29 DNA as indicated. Arrow shows the position of p5as- or p53-shifted DNA. Free probe is shown.

	PAb421	PAb246	PAb240	CM5	ApAs	PreI	IgG
TM3/19 (high)	neg	N-rare	neg	N~70%	N~2%	neg	neg
TM3/20 (high)	neg	N-rare	C~20%	N~60%	N~50%	neg	neg
TM10	N-rare	neg	neg	N~70%	C~10%	neg	neg

Table 1. Immunohistochemistry of TM3 (high passage) and TM10 tissue.

Percentages of cells reactive to each antibody are shown and represent an estimate of duplicate sections. PreI indicates preimmune serum. IgG was a mixture of IgG_1 and IgG_{2a}. N=nuclear, C=cytoplasmic, neg=negative.

		R/Y	F	I	#I	*
6	ATGGGGG**CATG**AGATTAAATAACCCAAATGAGGG**CATG**CGC**CATG**TCTGGCCC	+/-	-	-	23,3	-
13	AGCGTCAACAAGCGATTAGACACAC<u>GAA**CATG**TCCGGA**CATG**TTC</u>TAGCAAGAGCGTCT	+	+	+	6	-
14	ATGGGGG**CATG**AGATTAATAACCCAAATGAGGGCATCCG**CATGCATG**<u>CCT</u>CGGCCC	+/-	-	-	28,0	-
18	CCACCGGGA**CATG**TCGTT<u>*CTTG*</u>TTTTTCTGAATGGA<u>*CTTG*</u>TTCGCATAAAGAGCCTGG	-	NA	NA	NA	+
21	GCCAACGAGTGAACGGGCATCCG**CATG**TTAACGGTT<u>TTGGC</u>TTATCGCTGTGAT	-	NA	NA	NA	+
28/24	TATCACAGCGATAAGCCAAACCGATTAA**CATG**CG**CATG**<u>CCGGTT</u>CACT<u>*CTTG*</u>GCGG (54N PRIMER) CAGAAGACTG<u>CCCGGG</u>**CATG**TTCTACCAAACTGTTCCCCTTCCTCCTGTG	+/-	-	-	2,88	+
36	GCCAACGAGTGAACGGG**CATG**CG**CATG**TTAACGGA (18N PRIMER) GCCACGGACCTGATCACGGGTCTGTCTAAAG<u>CGGTT</u>TGAATCGT**CATG**GGTGAATGGTAA	-	-	-	2,70	+/-
59/42	GCCAACGAGTGAACGGG**CATG**GCG**CATG**TTAACGGT<u>TTGGC</u>TTATCGCTGTGGT	-	-	-	3	+
62	ACGTGTAA**CATG**<u>CCCGGG</u>ATGTTGTTCGAGCCCGAAGAGTCAAAGGGTTGAGA*CTAG*GC	-	NA	NA	NA	-
63	A**CATG**AACAGGTTTAAGGTTAATGA**CATG**<u>CCCGGG</u>**CATG**CCGCGGTCTTTCAGTAGGGT	-	-	+	20,6	-
64/38	AGCCGCT<u>*CTTG*</u>*CTAG*<u>AA**CATG**TCCGGA**CATG**TTC</u>GTGTGTCTATCG<u>*CTTG*</u>TTGACGCT	+	+	+	6	+

Table 2. Sequences retrieved by CASTing

Underlined sequences are motifs shared with known p53 binding sequences shown in Table 3. CATG sequences are in bold; C(A/T)(T/A)G sequences are in italics.

R/Y = + indicates 3 purines upstream and 3 pyrimidine downstream of CATGs; +/- indicates some, but not all, of the upstream or downstream sequences conform to 3 purines upstream and 3 pyrimidines downstream of CATGs; - indicates none of the upstream or downstream nucleotides conform to 3 purines upstream and 3 pyrimidines downstream of CATGs

F = 3 nucleotides flanking CATGs hybridize

I = intervening sequences (between CATGs) hybridize

#I = number of intervening nucleotides between CATG sequences

* = homology with MgBH6 (ref. 20) shown in Table 4

				53/p53as binding
1CATG	CAAACGCGTAGC	GGA**CATG**TCC	TGCGCGACAGGTCC	-
2CATG0	CAAACGCGTAGC	**CATGCATG**	TGCGCGACAGGTCC	-
2CATG1	CAAACGCGTAGC	**CATGCCATG**	TGCGCGACAGGTCC	-
2CATG3	CAAACGCGTAGC	**CATG**CCA**CATG**	TGCGCGACAGGTCC	-
2CATG6	CAAACGCGTAGC	GGA**CATG**TCCGGA**CATG**TCC	TGCGCGACAGGTCC	+
2CATG29	GGA**CATG**TCC	CAAACGCGTAGCTGCGCGACAGGTCC	GGA**CATG**TCC	+
SPLIT	CAAACGCGTAGC	**TG**TCCGGA**CA**	TGCGCGACAGGTCC	-
WAF-1	CAAACGCGTAGC	**CATG**TCCCAA**CATG**	TGCGCGACAGGTCC	+
mWAF-1		GAA**CATG**CCCAA**CATG**AA		+
CCCGGG	CAAACGCGTAGC	**CATG**CCCGGG	TGCGCGACAGGTCC	-

Table 3. Synthetic DNA sequence tested in gel shift assays with p53 and p53as proteins translated *in vitro*.

The flanking sequences used in all oligonucleotides were described previously (21). p53 was tested in the presence and absence of PAb421 and bound only in its presence. WAF-1 has the wild type intervening sequence but not the flanking sequences. mWAF-1 has changes in the binding sequence compared with WAF-1 (see Table 3). "Split" has a central portion of the cyclin G sequence (see Table 3). CCCGGG has only 1 CATG sequence.

		p53as binding	R/Y	F	I	#I	*
WAF-1	GAA**CATG**TCCCAA**CATG**TTG	+	+/-	-	-	6	-
P53CON	GAA**CATG**CCCGGG**CATG**TCC	+	+	+	+	6	-
MgBH6	GACACTGGTCACACTTGGCTGCTTAGGAAT	ND	NA	NA	NA	NA	+
CYCLIN G	AGGCCAGACCTGCCCGGGCAAGCCTTGGCA	+	NA	NA	NA	NA	+
RGC	GTTTGCCTTGCCTGGACTTGCCTGGCCTTGCCTT	+/-	NA	NA	NA	NA	+/-
50-2	TGGCAAGCCTATGA**CATG**GCCGGGGCCTGCCTCTCTCTGCCTCTGACCCT	ND	NA	NA	NA	NA	-

Table 4. Known p53 binding sequences.

CATGs are shown in bold; underlined sequences are motifs shared with CASTing sequences.

R/Y = + indicates 3 purines upstream and 3 pyrimidine downstream of CATGs; +/- indicates some, but not all, of the upstream or downstream sequences conform to 3 purines upstream and 3 pyrimidines downstream of CATGs;

F = 3 nucleotides flanking CATGs hybridize

I = intervening sequences (between CATGs) hybridize

#I = number of intervening nucleotides between CATG sequences

* = homology with MgBH6 (ref. 20)

Motif	Casting Sequences	Published Sequences	References
PuPuPuCA/TT/AGPyPyPy	13, 64/38		(19,22)
GAACATGTCCGGACATGTTC	13, 64/38		
CCCGGG	28/24, 62, 63	cyclin G, p53con	(16,22)
TTGGC	21, 28/24, 59/42 (inverted repeats)	MgBH6	(20)
CTTG	18, 28/24, 64/38)	cyclin G, RGC, MgBH5	(16,18,20)
ACTTG	18, 28/24 (inverted repeats)	RGC, MgBH6	(18,20)
TGGCT	21, 59/42	MgBH6	(20)
TGCCT	14	RGC, 50-2	(18,32)
TGCCC	28/24, 62, 63	cyclin G, p53con	(16,22)
TGTCC	13, 64/38	WAF-1, p53con	(13,22)
GGCAAGCCT	none	cyclin G, 50-2	(16,32)

Table 5. Summary of motifs and sequences in which each is found.

CONCLUSIONS

Task 1. We have completed determination of p53 and p53as expression in the mouse mammary model by carrying out immunohistochemistry on TM3 (high passage) and TM10 tissue and RT-PCR on RNA isolated from both cell lines. p53as and p53 mRNAs were present in all TM cell lines examined last year (TM3, TM4, TM9, and TM12) and in the two cell lines reported here, TM3 (high passage) and TM10. Both TM3 (high passage) and TM10 tissue contain p53 and p53as proteins, although in TM3 the proteins are not wild type. The genotype of p53 in TM10 tissue has not been determined since levels of protein were undetectable using PAb240 and PAb246.

Task 2. Production and purification of p53 and p53as proteins in the baculovirus system has been completed. Yields of antibody-affinity purified protein was ≤10 mg/50 ml culture and of nickel-affinity purified his-tagged proteins was ≥ 215 μg/50 ml culture. The his-tagged p53 or p53as proteins may be used as an alternative method to GST-fusion proteins to identify associated proteins as discussed in Task 6.

Task 3. This task was completed in Year 1 and showed that both *in vitro* translated p53 and p53as formed dimers and tetramers.

Task 4. We reported the half life of p53 and p53as in asynchronous TM3 low passage (nontumorigenic) and TM4 (highly tumorigenic) mammary cells in Progress Report 1995, and now report the p53 and p53as half life in asynchronous 291 (normal), and 291.05RAT (malignant) epidermal cells. Since both TM3 and TM4 contain a mutant p53 and p53as, these proteins were, as expected, long lived; in both TM3 and TM4 cells p53 had a half life of approximately 5 hours while p53as was approximately 6 hours. However, in 291 and 291.05RAT cells, both proteins were shorter lived as these cells contain a wild type protein. In fact, the half life was most likely increased because the cells had been exposed to the DNA damaging agent genistein to increase the amount of p53 and p53as and genistein has been shown to stabilize both proteins. In 291 cells the half life of p53 was 3 hours while p53as was 2.5 hours In 291.05RAT, the half life of p53 was 2.7

hours and of p53as 0.7 hour. The fact that in mammary cells p53as is more stable than p53 while it is less stable than p53 in the epidermal cells may be indicative of differences in cell type or differences between wild type and mutant proteins.

The increased stability of the mutated p53 and 53as proteins due to mutation in TM3 cells and the low levels of p53 and p53as proteins in TM10 cells would affect protein half-life studies. Therefore, we have chosen to concentrate on 291 epithelial cells which are a normal cell containing wild type p53. We have been unable to synchronize cells to perform half life studies of p53 and p53as during the stages of the cell cycle. However, in the coming year, we will explore the use of cell cycle arrest drugs to achieve synchronization. It has been observed that colon epithelial cells can be synchronized by amphidicolin, a G_1 arrest agent (Dr. J. Black, personal communication). This will be used as will higher doses of genistein, a G_2 arrest agent. We aim to block cells in G_1 or G_2 and test whether the half life of p53 or p53as is cell cycle dependent in these cell populations.

Task 5. We have completed this task with the finding that the DNA binding sequences of p53 and p53as are the same. The specificities of binding by either protein diverges from published consensus sequence of two copies of PuPuPuC(A/T)(T/A)GPyPyPy. We confirmed the central feature of the tetranucleotide CATG, but have observed a less rigorous requirement for flanking or intervening sequence than previously reported. In particular, the 3 upstream purines and 3 downstream pyrimidines are not required and 29 or more intervening nucleotides between the two motifs are tolerated. Moreover, one CATG is sufficient where adjacent nucleotides contain a region of homology with one or more previously reported nonconsensus p53 binding motifs.

Unique sequence motifs were identified that were found in known p53 binding sequences and CASTing sequences. These data suggest additional motifs and combinations of motifs with which to search a database for potential p53 target genes.

These results suggest that p53as and p53 exert their effect on the same target genes and that functional differences may be attributable to differential expression of the proteins or to differential regulation of their functions by modifications at either terminus by post-translational modifications and/or differential binding of associated proteins.

Task 6. Comparison between p53as- and p53-associated proteins has begun by construction of GST expression vectors containing the C-terminus of p53 or p53as. We have started to grow bacterial cultures containing large quantities of the fusion proteins in preparation for linking to glutathione beads and expect to have enough GST-p53as, GST-p53, and GST to link to beads in two months. The next approximately four months will be spent identifying associated proteins from 291 and TM10 cell extracts by affinity chromatography using these protein-bead complexes and examining bound proteins by SDS-PAGE and Coomassie staining. This procedure will be scaled up in order to obtain the amount of protein from bands of interest for microsequencing in the last 6 months of Year 3.

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